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Award Number: W81XWH-04-1-0386

TITLE: Role of the p53 Tumor Suppressor Homolog, p63, in Breast Cancer

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REPORT DATE: May 2005

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2005	Annual Summary (1 May 2004 - 30 Apr 2005)

4. TITLE AND SUBTITLE
Role of the p53 Tumor Suppressor Homolog, p63, in Breast Cancer

5. FUNDING NUMBERS
W81XWH-04-1-0386

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

p63 is a member of the p53 gene family, and shows structural and functional similarities to the p53 tumor suppressor. While p53's role in breast carcinogenesis is well established, p63's involvement in this disease remains unclear. It has been shown that p63 is expressed in the myoepithelial cells of the breast, and that p63 is essential for mammary development.

The main goal of this project is to investigate the potential role of p63 in breast cancer. Despite the homology to p53, p63's functions and mechanisms cannot necessarily be extrapolated from p53 paradigms. A key goal of this proposal is to compare and understand the mechanisms of transcriptional regulation by p63 versus p53. Do p53 and p63 have distinct or overlapping targets? We have made significant progress towards identifying p63 DNA-binding sites. We are also developing siRNA systems for analyzing the consequences of p63 depletion. Together, the proposed studies should offer a comprehensive view of transcriptional regulation and DNA binding by the p53 gene family. We anticipate that these data will help us understand their individual and interactive functions, as well provide important insights into signaling pathways in cancer and development.

14. SUBJECT TERMS
p53, p63, p73, tumor suppressors, transcription factors, gene regulation, mammary myoepithelial cells, mammary progenitors, chromatin immunoprecipitation

15. NUMBER OF PAGES
9

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction:

p63 is a member of the p53 gene family, and shows structural and functional similarities to the p53 tumor suppressor [1,2]. While p53's role in breast carcinogenesis is well established, p63's involvement in this disease remains unclear [3-6]. It has been shown that p63 is expressed in the myoepithelial cells of the breast, and that p63 is essential for mammary development.

The main goal of this project is to investigate the potential role of p63 in breast cancer. Despite the homology to p53, p63's functions and mechanisms cannot necessarily be extrapolated from p53 paradigms. Importantly, the p63 gene encodes multiple isoforms that can either mimic or antagonize p53 activity. It will therefore be important to understand the activities of individual p63 isoforms, as well their interactions with each other, and with p53. A key goal of this proposal is to compare and understand the mechanisms of transcriptional regulation by p63 versus p53. Do p53 and p63 have distinct or overlapping targets? What determines recruitment to their respective target sites? Do p53 and p63 cooperate or interfere with each other's transcriptional activity? While the proposed studies seek to address these issues specifically in breast tumorigenesis, it is likely that insights into p63 will have implications for cancer biology as a whole.

Body:

During this project period, we prioritized aims described in Task 2 (Statement of Work). Specifically, we used chromatin immunoprecipitation (ChIP) and genomic microarrays -- 'ChIP on chip' – to identify *in vivo* transcriptional targets for p63, as well as for the other two members of the gene family, p53 and p73. This was based on immediate opportunities presented by our collaboration with Affymetrix (Santa Clara, CA), and the development of mammalian ChIP protocols in our lab.

While the original proposal suggested the use of mammary cell lines (e.g. MCF-10A) in the ChIP on chip experiments, we decided to use an alternate cell line, ME180, for these large-scale preps. Several reasons led to this decision: 1) there is considerably lower expression of p63 in MCF-10A versus ME180 – we felt that abundant expression of protein would facilitate immunoprecipitation and subsequent detection of DNA binding sites on the genomic arrays. 2) Prior to the proposal being funded, we had initiated work on ME180 cells, and had made more progress in optimizing conditions for the ChIP on chip experiments. Overall, we felt we should proceed with ME180 as a 'workhorse' cell line for the large-scale experiments. More defined, downstream analysis may then be performed in mammary epithelial or cancer cell lines.

1. Purification and Identification of p53, p63, and p73 DNA binding sites

Large-scale chromatin immunoprecipitations (ChIP) were performed on ME180 cervical carcinoma cells. This cell line was chosen primarily because it expresses detectable – albeit non-equivalent – levels of p63, p73, and wildtype p53. We compared ME180 cells in the presence or absence of 5nM actinomycin D (ActD), a genotoxic drug shown to induce p53 expression. Chromatin was prepared by using micrococcal nuclease digestion and sonication to generate DNA fragments in the 500-1500bp range. For each immunoprecipitation (IP), chromatin from ~5 X 10^7 cells was used. Antibodies to p53, p63, or p73 were pre-coupled to protein A/G sepharose beads, and then used to immunoprecipitate crosslinked protein-DNA complexes. The resulting immunoprecipitates were treated with pronase to remove bound proteins, and heated to reverse crosslinking.

Quantitative polymerase chain reaction (qPCR) was used to confirm the success of the immunoprecipitations. Specifically, we looked for enrichment of a p53-binding site on the p21/Waf gene, a known target for p53, p63, and p73. We also tested a region on the JAG1 gene that has been reported to be a target for p63. We were able to demonstrate significant enrichment of the p21 and JAG1 sites for p63, and, to a lesser extent, p73 and p53. In order to obtain sufficient material for microarray hybridizations, we amplified the IP DNA using random primers in two successive primer extension reactions followed by PCR. This typically generates at least 20 ug of amplified DNA, sufficient for all the microarrays we intend to use. Importantly, the amplified DNA is also subjected to qPCR validation on the p21 and JAG1 sites.

Three independent biological replicates were performed, and the resulting amplified DNAs were sent to Affymetrix for hybridization to their human genome arrays. At present, the DNA samples have been hybridized to the ENCyclopedia Of DNA Elements (ENCODE) array. The ENCODE array is comprised of small regions of 0.5 to 2 megabases (Mb) chosen by an international consortium. Criteria for selection included well-annotated regions of the genome and availability of comparative sequence data. In sum, the ENCODE array encompasses about 30 Mb, or 1%, of the human genome, and contains roughly 300 annotated genes. While this array is by no means a comprehensive view of the entire genome, it offers a range of genomic features on a single array. This allowed us to probe a variety of regions, and further served as a 'proof of principle' and quality control experiment for the mammalian ChIP-chip technology.

Data from the three biological replicates show promising, highly reproducible results, especially for p63 binding sites. Using a stringent statistical cut-off of p-value (p-val) less than $1x10^{-5}$, we identified about 30 to 50 sites for p63 in each biological replicate. Significantly fewer sites were found for p73, and almost none were obtained for p53 in a reproducible manner. Given that three biological replicates were performed, we were able to use a combined p-value approach to identify bindings sites that consistently appear in all three experiments, despite failing the initial p-value cut-off. This analysis yielded approximately 100 sites for p63, between 10 and 40 for p73, and fewer than 15 for p53.

Several reasons may explain the discrepancy in number of binding sites for these factors: 1) Antibody efficiency represents a technical but important variable in these ChIP experiments. Preliminary western blotting results indicate that the p73 and p53 antibodies do not perform as well as the p63 monoclonal under present immunoprecipitation conditions. 2) Relative expression levels of p53 vs. p63 vs p73. It appears that ME180 cells express higher levels of p63 than p73 or p53 protein. While a precise, quantitative comparison is still pending, p63 levels are higher in ME180 than other cell lines tested (e.g. keratinocytes, mammary epithelial cells). Therefore, one reason we observe many more sites for p63 may be that it is, in relative terms, 'overexpressed' in these cells. 3) Biased representation of binding sites on the ENCODE array. As the ENCODE array contains only 1% of the genome, it is possible that p53 and p73 sites are excluded, while p63 targets are preferentially represented. Since so few targets are known for p63 and p73, it is difficult to confirm this hypothesis. Bias toward p63 sites would be a fortuitous and unintended phenomenon, but underscore the limitations of surveying such a small portion of the genome. 4) Finally, it remains possible that our ENCODE data accurately reflects the predominance of p63 binding sites in the genome. As noted, p63 is highly expressed under basal conditions – an observation that may allude to its role as a more global DNA binding factor.

2. Analysis of p63 binding sites from the ENCODE Array

The p63 ENCODE data generated the highest level of confidence based on reproducibility and overlap across independent experiments. We have therefore performed additional analysis and bioinformatics on the 100 or so sites identified.

(a) Overlap of p63 binding sites in (+) vs (-) Actinomycin D samples

The use of ActD, a genotoxic agent, was chosen largely to induce p53 expression. Based on western blot analysis, p63 and p73 levels did not change significantly in response to ActD. Accordingly, we found a strong overlap (~75%) of p63 binding sites for the (+) and (-) ActD samples, based on individual and combined p-values in all three biological replicates. This supports the notion that ActD did not appreciably alter p63's activity, in contrast to the increased DNA binding that is typically observed for wildtype p53 in response to genotoxic stimuli. Moreover, the similarity between the (+) and (-) ActD samples essentially means that six biological replicates were performed for p63. The degree of overlap in these six experiments was remarkable, and further increases our confidence that bona fide targets for p63 have been identified.

(b) p53 consensus motif in p63 binding sites

Given the strong sequence similarity (~65% identity) between the DNA binding domains of p63 and p53, we asked whether p53 consensus binding motifs could be found in the p63 binding sites on the ENCODE array. The p53 consensus is comprised of two, 10bp repeats (5'-PuPuPuC(A/T)(T/A)GPyPyPy-3') separated by a spacer of 0-13 bp. Using this consensus, and allowing for up to 3 mismatches except at the invariant C and G positions, we determined that 55 (out of 117) and 46 (out of 94) binding sites in the (-) and (+) ActD p63 samples, respectively, contained one or more copies of the p53 motif. The p-values for these observations were 5.2E-6 and 6.3E-7, respectively, for (-) and (+) ActD. Thus, roughly 50% of p63 targets contain the p53 consensus. Efforts are underway to determine whether the remaining 50% contain a novel motif for p63 binding.

(c) Location and characterization of p63 binding sites

p63 is presumed to behave, like p53, as a sequence specific transcription factor. Therefore, we analyzed the location of p63 binding sites to determine whether these could be associated with known genes or expressed sequences. A binding site was considered to be 'associated' with a gene if it resided up to 5 kilobases (Kb) upsteam (or 5') of the transcriptional start site, within the gene itself (exons and introns), OR was found within 1 Kb downstream of the gene. Using these parameters, we found that over 50% of p63 binding sites are associated with genes annotated as 'full-length', while up to 80% are in the proximity of full-length genes, mRNAs, and expressed sequence tags (ESTs) combined. p63's presence at these loci suggests its role in regulating transcription of the associated genes.

We then looked at the specific location of p63 binding at these associated genes. That is, does p63 bind within the canonical, upstream promoter region? Or does p63 exert its effects in a more long-range fashion from elsewhere in the gene? A detailed analysis was possible only for the 50% of p63 binding sites associated with full-length mRNAs. Here, we found that p63 bound predominantly in intronic regions (~70% of total sites), particularly intron 1 (~50% of intronic sites). The remaining p63 sites were distributed across the 5' upstream region (~20-30%), and few sites were found in the 3' downstream and exonic regions (less than 5% each). These data suggest that p63-responsive elements reside largely within introns, supporting the

notion that transcription factor binding sites are not restricted to the 5' region immediately upstream of transcribed genes, particularly in mammalian genomes.

3. Quantitative PCR validation of p63 binding sites

An important step towards confirming the binding sites from these ChIP-Chip experiments is the use of quantitative PCR to assess enrichment of target sequences. Specifically, we analyze the unamplified DNA material from the immunoprecipitations, so as to avoid – and simultaneously reveal -- potential artifacts from the amplification procedure. We chose over 10 loci identified as p63 targets and performed qPCR using real-time detection of SYBR green labeling. These experiments showed that targets identified by the cut-off p-value > 10E-5 typically showed 10-to over 100 fold enrichment in the p63 samples, relative to a reference (i.e. presumably non-enriched) region. This enrichment was specific for the p63 samples (plus and minus ActD), as DNA from immunoprecipitations with anti-p53 or anti-HA epitope antibodies failed to show enrichment. Of note, the p73 IPs frequently showed small to moderate enrichment for p63 target sequences. This finding supports the notion that p73 binds at least some of the same sites as p63. It is unclear at present whether the differences in fold enrichment between p63 and p73 reflect IP efficiency, protein expression levels, or true occupancy status.

Together, these data validate the large-scale, mammalian ChIP-Chip technology we have developed. We are working toward improving statistical tools for analyzing data and increasing our ability to address biologically meaningful questions.

4. Expression levels of p53 family members

We have initiated efforts toward a quantitative analysis of p53, p63, and p73 expression in ME180 and other cell types. This is aimed at probing mechanisms of action as well as functional interactions among the family members. Do relative expression levels explain the predominance of p63 targets versus p73 or p53? Do the family members compete with one another on various targets? For instance, if p63 levels are lowered, would we see a resulting increase in p53 or p73 DNA binding? Lastly, we wish to understand the relative expression levels of the multiple isoforms encoded by the p63 and p73 genes. This analysis may be useful in determining whether these factors behave as activators or repressors of gene transcription, as well as assessing the relative contribution of each isoform.

(a) Western blotting analysis of p53 and p63 expression in ME180 cells

To determine the relative levels of protein expression in a quantitative manner, we used recombinant protein of known concentration as standards in western blots. This avoids variations in western blot signal intensity due to antibody affinity rather than actual protein amounts. At present, we are able to do this for p53 and p63 only, as recombinant p73 protein is not yet available. Accordingly, ME180 lysates were probed with anti-p53 or anti-p63 antibodies. At a first approximation, we detected ~1 ng of p63 in 125 ug total protein. p53 was present at much lower levels, with less than 100 pg detected in the same lysate. These results support the notion that p63 is present at considerably higher levels in ME180 cells, and may in part explain the prevalence of p63 vs. p53 binding sites.

(b) Analysis of p63 isoform expression

We first used immunoblotting with an anti-p63 antibody that recognizes all isoforms (4A4 clone), and then compared the same ME180 lysates with a TAp63-specific antibody (6E6 clone). The TA-specific antibody failed to detect p63 in ME180 cells, while the pan-p63 antibody showed expression given the same amount of total protein. These results indicate that ΔNp63 isoforms are the predominant class in ME180 cells.

We also looked at p63 expression at the RNA level. Total RNA was isolated from ME180 cells and first strand synthesis was performed with reverse transcriptase using an oligo d(T) primer. The resulting cDNA was subjected to quantitative PCR using primers specific for TA vs. ΔNp63. These experiments also showed higher levels of ΔNp63 than TAp63.

Together, these data indicate that non-transactivating p63 isoforms are predominant in ME180 cells, and highlight the possibility that p63 behaves as a transcriptional repressor at the binding sites we identified. This hypothesis may be further tested using strategies to overexpress or deplete specific p63 isoforms, and assessing the consequences on expression of these target genes.

Key Research Accomplishments:

development and optimization of mammalian ChIP-chip protocols
identification and characterization of p63 DNA binding sites in untreated vs. DNA-damaged cells
facilitate development of data analysis for genomic arrays
expression analysis of p63 isoforms

Reportable Outcomes:

Gingeras, T.R., D. Kampa, E.A. Sekinger, J. Cheng, H. Hirsch, S. Ghosh, Z. Zhu, S. Patel, A. Piccolboni, A. Yang, H. Tammana, S. Bekiranov, P. Kapranov, R. Harrison, G. Church, K. Struhl, *et. al.*, (ENCODE Consortium). (2004) The ENCODE (ENCyclopedia Of DNA Elements) Project. *Science* **306**:636-640

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Conclusions:

We have made significant progress towards identifying p63 DNA-binding sites. ChIP-chip experiments with whole genome arrays are currently underway. We are also developing siRNA systems for analyzing the consequences of p63 depletion. Together, the proposed studies should offer a comprehensive view of transcriptional regulation and DNA binding by the p53 gene family. We anticipate that these data will help us understand their individual and interactive functions, as well provide important insights into signaling pathways in cancer and development.

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